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GENETIC METHOD FOR THE EXPRESSION OF POLYPROTEINS IN PLANTS

The present invention relates to a method for increasing protein expression levels, in particular by the coexpression of two or more proteins in plants within a single transcription unit, to the coexpression and secretion of two or more proteins in plants, to linker sequences for use in the method of the invention, to DNA constructs for use in the invention and to plants transformed with the constructs of the invention.

For many applications based on genetic modification of plants by transgenesis, it is desirable to express co-ordinately two or more transgenes. For instance, coexpression in plants of transgenes encoding antimicrobial proteins with different biochemical targets can result in enhanced disease resistance levels, resistance against a broader range of pathogens, or resistance that is more difficult to overcome by mutational adaptation of pathogens. Other examples include those aimed at producing a particular metabolite in transgenic plants by coexpression of multiple transgenes that are involved in a biosynthetic pathway. There are different ways to obtain transgenic plants expressing multiple transgenes. One frequently chosen option is to introduce each transgene individually via separate transformation events and to cross the different single-transgene expressing lines. The drawback of this method is that the different transgenes in the resulting progeny will be inserted at different loci, which complicates the subsequent breeding process. Moreover, this method is not applicable to crops that are propagated vegetatively, such as for instance potato, many ornamentals and fruit tree species.

A second possibility is to introduce the different transgenes as linked expression cassettes, each with their own promoters and terminators, within a single transformation vector. Such a set of transgenes will in this case segregate as a single genetic locus. It has been observed, however, that the presence of multiple copies of the same promoter within a transgenic plant often results in transcriptional silencing of the transgenes (Matzke, M.A. and Matzke, A.J.M., 1998, Cellular and Molecular Life Sciences 54, 94-103). In an attempt to introduce a vector containing four linked transgenes each driven by a CaMV35S promoter, Van den Elzen P.J. et al. (Phil. Trans. R. Soc. Lon. B., 1993, 342: 271-278) observed that none of the analysed transgenic lines expressed all four transgenes at a reasonably high level. To avoid this problem one could use different promoters for each of the expression cassettes used

in the construct. However, there is currently only a very limited choice of promoter sets that have comparable characteristics in terms of expression levels, cell-type and developmental specificity and response to environmental factors.

A third option would be to produce multiple proteins from one transcription unit by separating the distinct coding regions by so-called internal ribosomal entry sites, which allow ribosomes to reiterate translation at internal positions within a mRNA species. Although internal ribosomal entry sites are well documented in animal systems (Kaminski A. et al., 1994, Genet. Eng. 16, 115-155) it is not known at present whether such sites are also functional in nuclear-encoded genes from plants. Polycistronic genes can be expressed when inserted in plant chloroplastic genomes (Daniell H. et al., 1998, Nature Biotechnology 16, 345-348) but the gene products in this case are confined to the chloroplast, which is not always the preferred site of deposition of foreign proteins.

A fourth strategy, finally, is based on the production of multiple proteins by proteolytic cleavage of a single polyprotein precursor encoded by a single transcription unit. Potyviruses, for instance, translate their genomic RNA into a single polyprotein precursor that encompasses proteolytic domains able to cleave the polyprotein precursor in cis (Dougherty, W.G. and Carrington, J.C., 1988, Annu. Rev. Phytopathol. 26, 123-143). Beck von Bodman, S. et al., (1995, Bio/Technology 13, 587-591) have already exploited the potyviral system to co-express two enzymes involved in the biosynthesis of mannopine. The two biosynthetic enzymes were fused within one open reading frame together with a protease derived from a potyviral polyprotein precursor, and the adjoining regions were separated by 8 amino acids long spacers representing specific cleavage sites for the protease. Plants transformed with this construct synthesized mannopine, suggesting that the two enzymes had somehow been produced in a form that was at least partially functional, although direct evidence for the presumed cleavage events in planta was not presented. A disadvantage of this system is that a viral protein needs to be co-expressed with proteins of interest, which is not always desirable. More recently, Urwin P.E. et al. (1998, Planta 204, 472-479) have shown that it is possible to co-express two different proteinase inhibitors joined by a protease-sensitive propeptide derived from a plant metallothionein-like protein. A polyprotein precursor consisting of a cysteine protease inhibitor (oryzacystatin from rice), a propeptide from pea metallothionein-like protein and a serine protease inhibitor (cowpea trypsin inhibitor), was found to be cleaved in transgenic

Arabidopsis thaliana plants. The cleavage, however, was only partial, as uncleaved polyprotein precursor could also be detected in the transgenic plants. As the polyprotein precursor did not contain a leader peptide, the translation products are predicted to be deposited in the cytosol. The metallothionein from which the propeptide was derived also does not contain a leader peptide (Evans IM 1990, FEBS Lett. 262, 29-32) and hence its processing must occur in the cytosol.

For some applications, cytosolic processing and deposition is a drawback. Many proteins, especially glycosylated proteins or proteins with multiple disulfide bridges, must be synthesized in the secretory pathway (encompassing the endoplasmic reticulum and Golgi apparatus) in order to be folded in a functional form (Bednarek and Raikhel 1992, Plant Mol. Biol. 20, 133-150). In addition, for some applications such as for instance the expression of antimicrobial proteins, the extracellular space is the preferred deposition site, as most microorganisms occur at least during the early stages of infection in the extracellular space. Proteins destined to the extracellular space are also synthesised via the secretory pathway but lack additional targeting information other than the leader peptide (Bednarek and Raikhel 1992, Plant Mol. Biol. 20, 133-150). Other examples of the application of this strategy are described in WO 95/24486 and WO95/17514.

The applicants have unexpectedly found that expression levels of plant defensins in plants transformed with a polyprotein precursor construct were much higher compared to those in plants transformed with single plant defensin constructs.

The invention therefore provides a method of improving expression levels of a protein in a transgenic plant comprising inserting into the genome of said plant a DNA sequence comprising a promoter region operably linked to two or more protein encoding regions and a 3'-terminator region wherein said protein encoding regions are separated from each other by a DNA sequence coding for a linker propeptide said propeptide providing a cleavage site whereby the expressed polyprotein is post-translationally processed into the component protein molecules.

The processing system described here can be used not only to co-express two or more different proteins, but also to obtain higher expression levels of a protein, particularly of small proteins. The reason for the observed stimulatory effect on translational efficiency is currently

unclear. It might be due to an effect of mRNA length or length of primary translation product on translational efficiency.

Preferably, a signal sequence is operatively interconnected with the protein coding regions.

As used herein the expression "signal sequence" is used to define a sequence encoding a leader peptide that allows a nascent polypeptide to enter the endoplasmic reticulum and is removed after this translocation.

The signal sequence may be derived from any suitable source and may for example be naturally associated with the promoter to which it is operably linked. We have found the use of signal sequences from the class of plant proteins known as defensins (Broekaert et al, 1995 Plant Physiol 108, 1353-1358; Broekaert et al, 1997, Crit, Rev, Plant Sci. 16, 297-323) to be particularly suitable for use in the method of the invention.

Thus, in a further preferred embodiment, there is provided a method of improving expression levels of a protein in a transgenic plant comprising inserting into the genome of said plant a DNA sequence comprising a promoter region operably linked to a signal sequence said signal sequence being operably linked to two or more protein encoding regions and a 3'-terminator region wherein said protein encoding regions are separated from each other by a DNA sequence coding for a linker propeptide said propeptide providing a cleavage site whereby the expressed polyprotein is post-translationally processed into the component protein molecules.

This method of the invention is particularly suitable for the expression of proteins which are 100 amino acids or less in length

The present invention provides a convenient and highly efficient method of co-expressing two or more proteins in a plant as a single transcription unit where the two proteins are joined by a cleavable linker, the construct being designed such that cleavage occurs in the secretory pathway of the plant thereby releasing the proteins extracellularly.

According to a further aspect of the present invention, there is provided a method for the expression of multiple proteins in a transgenic plant comprising inserting into the genome of said plant a DNA sequence comprising a promoter region operably linked to a signal sequence said signal sequence being operably linked to two or more protein encoding regions and a 3'-terminator region wherein said protein encoding regions are separated from each

other by a DNA sequence coding for a linker propeptide said propeptide providing a cleavage site whereby the expressed polyprotein is post-translationally processed into the component protein molecules.

The two or more protein encoding regions according to all aspects of the invention preferably do not encode identical proteins i.e. the method of the invention allows the production of different proteins in a single transcription unit. The DNA sequence to be expressed according to the method of the invention is one which does not occur naturally in the plant used for the production of the multiple proteins i.e. one or more of the components of the DNA sequence will be heterologous to the plant host.

The method for the expression of multiple proteins described herein does not cover the use of a linker propeptide as expressed by the Ib-AMP gene and as described in SEQ ID Nos 14,15, 16, 17 or 18 of Published International Patent Application No. WO 95/24486 separating three protein encoding regions each of which encodes Rs-AFP2; nor the insertion thereof into a plant genome. Suitably, the method of the invention does not use a linker propetide of the native Ib-AMP gene as shown in SEQ ID Nos 14, 15, 16, 17 or 18 of WO 95/24486.

In a further aspect, the present invention there is provided a method for the expression of multiple proteins in a transgenic plant comprising inserting into the genome of said plant a DNA sequence comprising a promoter region operably linked to a signal sequence said signal sequence being operably linked to two or more protein encoding regions and a 3'-terminator region wherein said protein encoding regions are separated from each other by a DNA sequence coding for a linker propeptide said propeptide providing a cleavage site whereby the expressed polyprotein is post-translationally processed into the component protein molecules with the proviso that when the linker propeptide is derived from the Ib-AMP gene as described in SEQ ID Nos 14,15, 16, 17 or 18 of Published International Patent Application No. WO 95/24486 it does not separate three protein encoding regions each of which encodes Rs-AFP2.

The sequence of Rs-AFP2 is fully described in Published International patent Application no. WO 93/05153 published 18 March 1993.

The promoter sequence may for example be that naturally associated with the signal sequence, and/or it may be that naturally associated with the protein encoding sequence to which it is linked, or it may be any other promoter sequence conferring transcription in plants. It may be a constitutive promoter or it may be an inducible promoter.

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The linker propeptide for use in all aspects and embodiments of the invention described herein is preferably a linker propeptide which is cleaved on passage of said DNA encoding the polyprotein precursor through the secretory pathway of the plant cells in which the polyprotein -encoding DNA is expressed. The linker propeptide is preferably designed or
5   chosen such that cleavage of the propeptide occurs by proteases which are naturally present in the secretory pathway of the plant cell in which the DNA encoding the polyprotein is expressed. Particular promoters of the cauliflower mosaic virus such as the Penh 25S promoter of the 35S RNA, examples of such proteases include subtilisin-like proteases, .

In a preferred embodiment the invention therefore provides a method for the
10   expression of multiple proteins in a transgenic plant comprising inserting into the genome of said plant a DNA sequence comprising a promoter region operably linked to a signal sequence, said signal sequence being operably linked to two or more protein encoding regions and a 3'-terminator region wherein said protein encoding regions are separated from each other by a DNA sequence coding for a linker propeptide said propeptide providing a cleavage
15   site whereby the expressed polyprotein is post-translationally processed into the component protein molecules said linker propeptide being cleaved on passage of said DNA encoding the polyprotein precursor through the secretory pathway of the plant cells in which the polyprotein -encoding DNA is expressed.

The method for the expression of multiple proteins described herein does not cover the
20   use of a linker propeptide derived from the Ib-AMP gene as described in SEQ ID Nos 14, 15, 16, 17 or 18 of Published International Patent Application No. WO 95/24486 separating three protein encoding regions each of which encodes Rs-AFP2 and the insertion thereof into a plant genome.

In some embodiments of the invention, the linker propeptide is not derived from a
25   virus.

In a particularly preferred embodiment the invention provides a method for the expression of multiple proteins in a transgenic plant comprising inserting into the genome of said plant a DNA sequence comprising a promoter region operably linked to a signal sequence said signal sequence being operably linked to two or more protein encoding regions and a 3'-
30   terminator region wherein said protein encoding regions are separated from each other by a DNA sequence coding for a linker propeptide said propeptide providing a cleavage site

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whereby the expressed polyprotein is post-translationally processed into the component protein molecules. said linker propeptide being cleaved on passage of said DNA encoding the polyprotein precursor through the secretory pathway of the plant cells in which the polyprotein -encoding DNA is expressed wherein cleavage of the propeptide occurs by proteases which are

5   naturally present in the secretory pathway of said plant cell.

The linker propeptide may be a peptide which naturally contains processing sites for proteases occuring in the secretory pathway of plants such as the internal propeptides derived from the Ib-AMP gene which are described further herein, or may be a peptide to which such a protease processing site has been engineered at either or both ends thereof to facilitate

10   cleavage of the sequence. Where a propeptide possesses one such protease processing site a further protease processing site may be added. If necessary or desired, repeats of the processing site, for example up to 6 repeats may be included.

For example, as described fully herein, a further protease processing site has been added to the 3' end of the DNA sequence coding for the C-terminal propeptides from Dahlia

15   and Amaranthus which naturally possess a protease processing site at their N-terminal end for an unknown secretory pathway protease and these peptides are particularly suitable for use according to the method of the invention. Certain Dahlia sequences including C-terminal propeptide sequences are described and claimed in copending British Patent Application No. 9818003.7.

20   Yet another strategy is based upon the use of virus e.g. picornovirus sequences such as 20 amino acid sequences called the 2A sequence of the foot-and-mouth disease virus (FMDV) RNA, which results in the cleavage of polyproteins (Ryan and Drew 1994, EMPO J., 13, 928-933). In this instance however, in order to avoid the retention of unwanted amino acids on the protein product, combined with a sequence which produces N-terminal

25   sequence, for example a plant derived sequence or a fragment thereof, to form a chimeric propeptide.

In the present invention. we have developed novel strategies for making artificial polyprotein precursors which are cleaved in the secretory pathway. The first one was based on the use of a propeptide derived from the IbAMP gene. IbAMP is a gene from the plant

30   *Impatiens balsamina* which encodes a peculiar polyprotein precursor featuring a leader peptide and six consecutive antimicrobial peptides. each flanked by propeptides ranging from 16 to 28

amino acids in length (Tailor R.H. *et al.*, 1997, J. Biol. Chem. 272, 24480-24487). It is not known how and where processing of the IbAMP precursor occurs in its plant of origin. One of the internal propeptides from IbAMP was used to separate two distinct plant defensin coding regions, one originating from radish seed (RsAFP2, Terras F.R.G. *et al.*, 1992, J. Biol. Chem.

5   267, 15301-15309; Terras et al 1995 Plant Cell, 7, 573-588) and one from dahlia seed (DmAMP1, Osborn R.W. *et al.*, 1995, FEBS Lett. 368, 257-262).

An other strategy was based on the use of C-terminal propeptides from either the DmAMP1 precursor or the AcAMP2 precursor (De Bolle M.F.C. *et al.*, 1993, Plant Mol. Biol. 22, 1187-1190) or fragments of these. These C-terminal propeptides were chosen based on

10   our previous observation that they apparently can be cleaved in transgenic tobacco plants without influencing extracellular deposition of the mature proteins to which they are connected in the precursor (R.W. Osborn and S. Attenborough, personal communication; De Bolle M.F.C. *et al.*, 1996, Plant Mol. Biol. 31, 993-1008) implicating that such cleavage is performed by a protease present in the secretory pathway excluding the vacuole. To convert

15   these C-terminal propeptides to internal propeptides, a subtilisin-like protease processing site was engineered at the C-terminal part of the propeptides.

Subtilisin-like proteases are enzymes that specifically cleave at recognition sites of which the last two residues are basic (Barr, P.J., 1991, Cell 66, 1-3; Park C.M. *et al.*, 1994, Mol. Microbiol. 11, 155-164). Although subtilisin-like proteases are best documented in fungi

20   (e.g. Kex2-like proteases) and higher animals (e.g. furins), recent evidence suggests that such enzymes are also present in plants (Kinal H. *et al.*, 1995, Plant Cell 7, 677-688; Tornero P. *et al.*, 1997, J. Biol. Chem. 272, 14412-14419), including Arabidopsis (Ribeiro A. *et al.*, 1995, Plant Cell 7, 785-794).

We have found that polyprotein precursors consisting of a leader peptide followed by

25   two different plant defensins separated from each other by any of the above described internal propeptides can be processed in transgenic plants to release both plant defensins simultaneously. The cleavage does occur such that at least the major part of the plant defensins are deposited in the extracellular space. Hence processing of the precursor occurred either in the secretory pathway or in the extracellular space. The different propeptides shown

30   to be cleaved in the transgenic plants do not reveal primary sequence homology. However, the sequences all appear to be rich in the small amino acids A, V, S and T and all contain

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dipeptidic sequences consisting of either two acidic residues, two basic residues or one acidic and one basic residue. Although propeptide cleavage in the examples shown in this invention did apparently not occur within vacuoles, internal propeptides from vacuolar proteins (e.g. 2S albumins) might also be used if vacuolar deposition of the proteins would be desirable. In the co-expression experiments described here two different plant defensins were used but it is predicted that similar results will be obtained when other types of proteins would be used or when more than two mature protein domains would be used in the polyprotein precursor structure.

Where it is desired to target the polyprotein to a particular cellular organelle along the secretory pathway a suitable targeting sequence may be added to one or more of the multiple protein encoding regions. For example, an endoplasmic reticulum targeting sequence such as that encoding KDEL (SEQ ID NO 65) may be added to the 3' end of one or more of the mature protein encoding regions, or a vacuolar targeting sequence (Chispeels and Raikhel 1992, Cell 68, 613-616) can be added to the 3' or 5' end of one or more of the protein encoding regions. An example of the latter is the barley lectin carboxy-terminal propeptide which has been shown to destine heterologous proteins that are otherwise secreted to the vacuoles (Bednarek and Raikhel 1991, Plant Cell 3, 1195-1206; De Bolle et al, 1996 Plant Mol. Biol. 31, 993-1008).

At least 40% of the sequence of the linker propeptide for use in accordance with all aspects and methods of the invention as described herein preferably consists of stretches of either two to five consecutive hydrophobic residues selected from alanine, valine, isoleucine, methionine, leucine, phenylalanine, tryptophan and tyrosine or stretches of two to five hydrophilic residues selected from aspartic acid, glutamic acid, lysine, arginine, histidine, serine, threonine, glutamine and asparagine.

The said hydrophobic residues are preferably alanine, valine, leucine, methionine and/or isoleucine and the said hydrophilic residues are preferably aspartic acid, glutamic acid, lysine and/or arginine.

It is further preferred that the linker propeptide has within 7 residues of its N- or C-terminal cleavage site a sequence with two to five consecutive acidic residues, two to five basic residues or two to five consecutive intermixed acidic and basic residues.

It is especially preferred that at least 40% of the sequence of the linker propeptide for use in accordance with all aspects of the invention as described herein preferably consists of stretches of either two to five consecutive hydrophobic residues selected from alanine, valine, isoleucine, methionine, leucine, phenylalanine, tryptophan and tyrosine or stretches of two to

5 five hydrophilic residues selected from aspartic acid, glutamic acid, lysine, arginine, histidine, serine, threonine, glutamine and asparagine and has within 7 residues of its N- or C- terminal cleavage site a sequence with two to five consecutive acidic residues, two to five basic residues or two to five consecutive intermixed acidic and basic residues.

The use of linker propeptides rich in the small amino acids A, V, S and T and

10 containing dipeptidic sequences consisting of either two acidic residues, two basic residues or one acidic and one basic residue which on translation provides a cleavage site whereby the expressed polyprotein is post-translationally processed into the component protein molecules is also preferred.

As used herein the term 'rich' is used to denote that the residues A,V, S and T are

15 present more frequently than would be expected based on a random distribution of amino acids.

It is further preferred that the linker propeptides have a dipeptidic sequence within seven amino acids from the N- and/or C- terminal ends thereof, the said dipeptidic sequences consisting of either two acidic residues, two basic residues or an acidic and a basic residue

20 wherein said dipeptidic sequences may be the same or different at each terminus.

In a further preferred embodiment said dipeptidic sequences are selected from the following EE, ED and/or KK.

It is particularly desirable that the linker propeptide should hold the two (or more) protein domains sufficiently far apart so that they can fold appropriately and independently.

25 For this purpose, the linker polypeptide is suitably at least 10 and preferably at least 15 amino acids long. It is further advantageous that the linker propeptide should not interact with any secondary structural element in the two proteins which it links and should therefore itself have no particular secondary structure or form a solitary secondary structure element such as an alpha helix.

30 In this and all other aspects and embodiments of the invention described herein the linker propeptide sequence providing the cleavage site preferably comprises a linker sequence

which is isolatable from a natural source such as a plant or virus, or variant thereof or a frament of either of these. In particular the linker propeptide is isolatable from a plant protein, or a fragment, or variant or derivative thereof which can provide suitable cleavage sites. Particular examples include a cleavable linker derived from the C-terminal propeptide region of a Dahlia gene such as those described and claimed in copending British Patent Application No. 9818003.7.

Where a viral sequence is used, it is preferably an element of a chimeric propeptide sequence.

The expression "variant" refers to sequences of amino acids which differ from the base sequence from which they are derived in that one or more amino acids within the sequence are substituted for other amino acids. Amino acid substitutions may be regarded as "conservative" where an amino acid is replaced with a different amino acid with broadly similar properties. Non-conservative substitutions are where amino acids are replaced with amino acids of a different type. Broadly speaking, fewer non-conservative substitutions will be possible without altering the biological activity of the polypeptide. Suitably variants have at least 85% similarity and preferably at least 90% similarity to the base sequence

In the context of the present invention, two amino acid sequences with at least 85% similarity to each other have at least 85% similar (identical or conservatively replaced) amino acid residues in a like position when aligned optimally allowing for up to 3 gaps, with the proviso that in respect of the gaps a total of not more than 15 amino acid residues is affected. Likewise, two amino acid sequences with at least 90% similarity to each other have at least 90% identical or conservatively replaced amino acid residues in a like position when aligned optimally allowing for up to 3 gaps with the proviso that in respect of the gaps a total of not more than 15 amino acid residues is affected.

For the purpose of the present invention, a conservative amino acid is defined as one which does not alter the activity/function of the protein when compared with the unmodified protein. In particular, conservative replacements may be made between amino acids within the following groups:

(i) Alanine, Serine, Glycine and Threonine

(ii) Glutamic acid and Aspartic acid

(iii) Arginine and Lysine

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(iv)   Isoleucine, Leucine, Valine and Methionine

(v)   Phenylalanine, Tyrosine and Tryptophan

        Sequence similarity may be calculated using sequence alignment algorithms known in the art such as, for example, the Clustal Method described by Myers and Miller

5   (Comput. Appl. Biosci .4 11-17 (1988).) and Wilbur and Lipman (Proc. Natl. Acad. Sci. USA 80, 726-30 (1983)) and the Watterman and Eggert method (The Journal of Molecular Biology (1987) 197, 723-728). The MegAlign Lipman Pearson one pair method (using default parameters) which may be obtained from DNAstar Inc, 1228 Selfpark Street, Madison, Wisconsin, 53715, USA as part of the Lasergene system may also be used.

10   In particular the linker propetide is a sequence isolatable from a plant protein and more preferably from the precursor of a plant antimicrobial protein such as a defensin. or a hevein-type antimicrobial peptide (Broekaert et al 1997, Crit. Rev. Plant Sci. 16, 297-323). The linker propeptide is most preferably derivable from a defensin and/or a hevein type antimicrobial peptide, especially from the C-terminal propeptides from Dm-AMP1 and Ac-AMP2 the

15   sequences of which are as described in Figure 2 herein (SEQ ID NO 5 and SEQ ID NO 8).

        The use of a linker propeptide derived from an antimicrobial peptide derived from the genus Impatiens is also preferred.   The Ib-AMP gene comprises five propeptide regions all of which are suitable for use in the present invention and which are described fully in Published International Patent Application WO 95/24486 at pages 29 and 40 to 42, the contents of

20   which are incorporated herein by reference. All or part of the C-terminal propeptides derived from the Dm-AMP and Ac-AMP gene may be used.

        In a particularly preferred embodiment, the linker propeptide sequence used comprises a naturally occurring linker propeptide sequence which is modified so that amino acids from said sequence remaining attached to protein product after cleavage thereof is reduced,

25   preferably so that none remain. Suitable modifications may be determined using routine methods as described hereinafter. In its simplest form, protein products of the invention are isolated and analyzed to see whether they include any residual amino acids derived from the propeptide linker. The linker sequence may then be modified to eliminate some or all of these residues, provided the function of post-translational cleavage remains.

The term "fragment" refers to sequences from which amino acids have been deleted, preferably from an end region thereof. Thus these include the modified forms of the natural sequences mentioned above.

A linker propeptide of the invention may comprise one or more such fragments from different sources provided it functions as a post-translational cleavage site. Examples of linker propeptide sequences are SEQ ID NOs 3, 4, 6, 7, 21, 22, 23, 24, 25, 26, 27, 28 and 29 as shown herein and variants therefore which act as a propeptide. Particular examples of these are SEQ ID NOs 3, 4, 6, 7, 21, 22, 23, 24, 25, 26, 27, 28 and 29 themselves.

In particular, the propeptide sequences comprise SEQ ID NOs 3, 4, 6 or 7.

According to a preferred embodiment the present invention further provides a method for the expression of multiple proteins in a transgenic plant comprising inserting into the genome of said plant a DNA sequence comprising a promoter region operably linked to a signal sequence said signal sequence being operably linked to two or more protein encoding regions and a 3'-terminator region wherein said protein encoding regions are separated from each other by a DNA sequence coding for a linker propeptide wherein the linker propeptide is derivable from a defensin and/or a hevein type antimicrobial peptide said propeptide providing a cleavage site whereby the expressed polyprotein is post-translationally processed into the component protein molecules.

The use of the C-terminal propeptides from Dm-AMP1 and Ac-AMP2 as described in Figure 2 herein as cleavable linkers i.e. to provide a cleavable linkage site, are particularly preferred. Depending on the choice of propeptide it may be necessary to engineer an additional specific protease recognition site at either or both ends to facilitate cleavage of the sequence. Suitable specific protease recognition sites include for example, recognition sites for subtilisin -like proteases recognising either a dipeptidic sequence consisting of two basic residues; tetrapeptidic sequences consisting of a hydrophobic residue, any residue, a basic residue and a basic residue or a tetrapeptidic sequence consisting of a basic residue, any residue, a basic residue and a basic residue. Subtilisin-like protease recognition sites are particularly preferred for use in the method of the invention.

According to a yet further preferred embodiment the present invention further provides a method for the expression of multiple proteins in a transgenic plant comprising inserting into the genome of said plant a DNA sequence comprising a promoter region operably linked to a

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signal sequence said signal sequence being operably linked to two or more protein encoding regions and a 3'-terminator region wherein said protein encoding regions are separated from each other by a DNA sequence coding for a linker propeptide said propeptide providing a cleavage site whereby the expressed polyprotein is post-translationally processed into the

5   component protein molecules and wherein an additional specific protease recognition site has been engineered at either or both ends of said linker propeptide to facilitate cleavage of the sequence.

According to a yet further preferred embodiment the present invention further provides a method for the expression of multiple proteins in a transgenic plant comprising inserting into

10   the genome of said plant a DNA sequence comprising a promoter region operably linked to a signal sequence said signal sequence being operably linked to two or more protein encoding regions and a 3'-terminator region wherein said protein encoding regions are separated from each other by a DNA sequence coding for a linker propeptide wherein the linker propeptide is derivable from a defensin and/or a hevein type antimicrobial peptide said propeptide providing

15   a cleavage site whereby the expressed polyprotein is post-translationally processed into the component protein molecules and wherein an additional specific protease recognition site has been engineered at either or both ends of said linker propeptide to facilitate cleavage of the sequence.

The invention further provides the use of propeptides isolatable from plant derived

20   proteins as cleavable linkers in polyprotein precursors synthesised via the secretory pathway in transgenic plants. The propeptides are preferably isolatable from the precursor of a plant defensin or a hevein-type antimicrobial peptide (Broekaert et al 1997, Crit. Rev. Plant Sci. 16, 297-323). The propeptides may also preferably be isolatable from an antimicrobial peptide derived from the genus Impatiens.

25   In a further aspect the invention provides the use of a propeptide wherein at least 40% of the sequence of the propeptide consists of stretches of either two to five consecutive hydrophobic residues selected from alanine, valine, isoleucine, methionine, leucine, phenylalanine, tryptophan and tyrosine or stretches of two to five hydrophilic residues selected from aspartic acid, glutamic acid, lysine, arginine, histidine, serine, threonine, glutamine and

30   asparagine as a cleavable linker in polyprotein precursors synthesised via the secretory pathway in transgenic plants.

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It is further preferred that the linker propeptide has within 7 residues of its N- or C- terminal cleavage site a sequence with two to five consecutive acidic residues, two to five basic residues or two to five consecutive intermixed acidic and basic residues.

It is especially preferred that at least 40% of the sequence of the linker propeptide consists of stretches of either two to five consecutive hydrophobic residues selected from alanine, valine, isoleucine, methionine, leucine, phenylalanine, tryptophan and tyrosine or stretches of two to five hydrophilic residues selected from aspartic acid, glutamic acid, lysine, arginine, histidine, serine, threonine, glutamine and asparagine and has within 7 residues of its N- or C- terminal cleavage site a sequence with two to five consecutive acidic residues, two to five basic residues or two to five consecutive intermixed acidic and basic residues.

In a further aspect the invention provides the use of a peptide sequence rich in the small amino acids A, V, S and T and containing dipeptidic sequences consisting of either two acidic residues, two basic residues or one acidic and one basic residue as a cleavable linker sequence wherein said sequence is isolatable from a plant defensin or a hevein-type antimicrobial protein.

The methods of the invention may be used to achieve efficient expression and secretion of any desired proteins and is particularly suitable for the expression of proteins which must naturally be synthesised in the secretory pathway in order to be folded in a functional form such as, for example, glycosylated proteins and those with disulphide bridges. Additionally, it is extremely advantageous for proteins involved in the defence of a plant to attack by a pathogen to be secreted efficiently to the extracellular space since this is usually the initial site of pathogen attack and the present methods of the invention provide an effective means of delivering multiple proteins extracellularly.

The method of the invention is also particularly suitable for producing small peptides which may then be used for immunisation purposes i.e. the transgenic plant or a seed derived therefrom may be used directly as a foodstuff thereby passively immunising the recipient.

Examples of proteins which may be expressed according to the methods of the present invention include, for example, antifungal proteins described in Published International Patent Application Nos WO92/15691, WO92/21699, WO93/05153, WO93/04586, WO94/11511, WO95/04754, WO95/18229, WO95/24486, WO97/21814 and WO97/21815 including Rs-AFP1, Rs-AFP2, Dm-AMP1, Dm-AMP2, Hs-AFP1, Ah-AMP1, Ct-AMP1, Ct-AMP2, Bn-

AFP1, Bn-AFP2, Br-AFP1, Br-AFP2, Sa-AFP1, Sa-AFP2, Cb-AMP1, Cb-AMP2, Ca-AMP1, Bm-AMP1, Ace-AMP1, Ac-AMP1, Ac-AMP2, Mj-AMP1, Mj-AMP2, Ib-AMP1, Ib-AMP2, Ib-AMP3, Ib-AMP4, PR-1 type proteins such as chitinases, glucanases such as beta1,3 and beta1,6 glucanases, chitin-binding lectins, zeamatins, osmotins, thionins and ribosome-
5 inactivating proteins and peptides derived therefrom or antifungal proteins showing 85% sequence identity, preferably greater than 90% sequence identity, more preferably greater than 95% sequence identity with any of said proteins where sequence identity is as defined above.

The cleavable linkers are used to join two or more proteins of interest and provide cleavage sites whereby the polyprotein is post-translationally processed into the component
10 protein molecules.

In a further aspect the invention provides a DNA construct comprising a DNA sequence comprising a promoter region operably linked to a plant derived signal sequence said signal sequence being operably linked to two or more protein encoding regions and a 3'-terminator region wherein said protein encoding regions are separated from each other by a
15 DNA sequence coding for a linker propeptide said propeptide providing a post-translational cleavage site.

Suitably the protein encoding region encode different proteins. Preferred examples of propeptide linker sequences are as detailed above.

In a preferred embodiment of this aspect the invention provides a DNA construct
20 wherein said DNA sequence encoding said linker propeptide encodes an internal propeptide from the Ib-AMP gene. In a further preferred embodiment of this aspect the invention provides a DNA construct wherein said DNA sequence encoding said linker propeptide encodes the C-terminal propeptide from the Dm-AMP or from the Ac-AMP gene.

In a particularly preferred embodiment the invention provides a DNA construct as
25 described above wherein when the DNA sequence encoding the linker propeptide is derived from the Dm-AMP gene or from the Ac-AMP gene it additionally comprises one or more protease recognition sites at either or both ends thereof.

In a further aspect the invention provides a DNA construct comprising a DNA sequence comprising a promoter region operably linked to two or more protein encoding
30 regions and a 3' terminator-region wherein said protein encoding regions are separated from each other by a DNA sequence coding for a linker propeptide encoding the C-terminal

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propeptide from the Dm-AMP gene or the from the Ac-AMP gene said propeptide providing a post-translational cleavage site.

In a particularly preferred embodiment the invention provides a DNA construct as described above wherein the DNA sequence encoding the linker propeptide from Dm-AMP or Ac-AMP additionally comprises one or more protease recognition sites at either or both ends thereof.

In a yet further aspect the invention provides a transgenic plant transformed with a DNA construct according to any of the above aspects of the invention.

In a further aspect the invention provides a transgenic plant transformed with a DNA sequence comprising a promoter region operably linked to a signal sequence said signal sequence being operably linked to two or more protein encoding regions and a 3'-terminator region wherein said protein encoding regions are separated from each other by a DNA sequence coding for a linker propeptide which on translation provides a cleavage site.

In a preferred embodiment of this aspect at least 40% of the sequence of the said linker propeptide consists of stretches of either two to five consecutive hydrophobic residues selected from alanine, valine, isoleucine, methionine, leucine, phenylalanine, tryptophan and tyrosine or stretches of two to five hydrophilic residues selected from aspartic acid, glutamic acid, lysine, arginine, histidine, serine, threonine, glutamine and asparagine.

The said hydrophobic residues are preferably alanine, valine, leucine, methionine and/or isoleucine and the said hydrophilic residues are preferably aspartic acid, glutamic acid, lysine and/or arginine.

It is further preferred that the linker propeptide has within 7 residues of its N- or C-terminal cleavage site a sequence with two to five consecutive acidic residues, two to five basic residues or two to five consecutive intermixed acidic and basic residues.

It is especially preferred that at least 40% of the sequence of the linker propeptide consists of stretches of either two to five consecutive hydrophobic residues selected from alanine, valine, isoleucine, methionine, leucine, phenylalanine, tryptophan and tyrosine or stretches of two to five hydrophilic residues selected from aspartic acid, glutamic acid, lysine, arginine, histidine, serine, threonine, glutamine and asparagine and has within 7 residues of its N- or C- terminal cleavage site a sequence with two to five consecutive acidic residues, two to five basic residues or two to five consecutive intermixed acidic and basic residues.

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In a further preferred embodiment of this aspect of the invention the DNA sequence providing the cleavage site encodes a peptide sequence rich in the small amino acids A, V, S and T and containing dipeptidic sequences consisting of either two acidic residues, two basic residues or one acidic and one basic residue.

5   In a particularly preferred embodiment of this aspect of the invention the DNA sequence providing the cleavage site encodes a propeptide derived from the Ib-AMP gene such as for example that described in Figure 2. In a further particularly preferred embodiment of this aspect of the invention the DNA sequence providing the cleavage site encodes the C-terminal propeptides from Dm-AMP1 and Ac-AMP2 as described in Figure 2 which may
10   optionally be engineered to include a further DNA sequence encoding a subtilisin-like protease recognition site.

In a further aspect the invention provides a vector comprising a DNA construct as described above.

Certain linker sequences described herein are novel and theses and the coding sequence
15   for these form a further aspect of the invention. In particular therefore, there is provided a nucleic acid which encodes a linker peptide of SEQ ID NO 4, 6, 7, 29, 21, 22, 23, 24, 25, 26, 27, 28 or the linker peptide shown in Figure 34 as well as variants thereof. Particular variants will be those which have SEQ ID NO 77 linked at the C-terminal end.

As will be readily apparent to a man skilled in the art the sequence of the individual
20   components of the DNA sequence i.e. the signal sequence, promoter sequence, linker sequence, protein sequence(s), terminator sequence for use in the methods according to the invention may be predicted from its known amino acid sequence and DNA encoding the protein may be manufactured using a standard nucleic acid synthesiser. Alternatively, DNA encoding the components of the invention may be produced by appropriate isolation from
25   natural sources.

The invention is further illustrated with reference to the following non-limiting examples and figures in which

Figure 1:   shows nucleotide sequence (SEQ ID NO 1) and corresponding amino acid sequence (SEQ ID NO 2) of coding region of the DmAMP1 gene. The amino acids
30   corresponding to mature DmAMP1 are underlined. The nucleotides corresponding to the intron are double underlined.

Figure 2:   shows schematic representation of the coding regions from the vector constructs (SEQ ID NOS 3-8). Amino acids sequences below the internal propeptides represent the propeptide sequences from which the linker propeptides were derived.

Figure 3:   shows schematic representation of plant transformation vector pFAJ3105

Figure 4:   shows schematic representation of plant transformation vector pFAJ3106

Figure 5:   shows schematic representation of plant transformation vector pFAJ3107

Figure 6:   shows schematic representation of plant transformation vector pFAJ3108

Figure 7:   shows schematic representation of plant transformation vector pFAJ3109

Figure 8:   shows nucleotide sequence (SEQ ID NO 9) and corresponding amino acid sequence (SEQ ID NO 10) of the open reading frame of the region comprised between the NcoI and SacI sites of plasmid pFAJ3105. The amino acids corresponding to mature DmAMP1 and mature RsAFP2 are underlined and double-underlined, respectively.

Figure 9:   shows nucleotide sequence (SEQ ID NO 11) and corresponding amino acid sequence (SEQ ID NO 12) of the open reading frame of the region comprised between the NcoI and SacI sites of plasmid pFAJ3106. The amino acids corresponding to mature DmAMP1 and mature RsAFP2 are underlined and double-underlined, respectively.

Figure 10: shows nucleotide sequence (SEQ ID NO 13) and corresponding amino acid sequence (SEQ ID NO 14) of the open reading frame of the region comprised between the NcoI and SacI sites of plasmid pFAJ3107. The amino acids corresponding to mature DmAMP1 and mature RsAFP2 are underlined and double-underlined, respectively.

Figure 11: shows nucleotide sequence (SEQ ID NO 15) and corresponding amino acid sequence (SEQ ID NO 16) of the open reading frame of the region comprised between the NcoI and SacI sites of plasmid pFAJ3108. The amino acids corresponding to mature DmAMP1 and mature RsAFP2 are underlined and double-underlined, respectively.

Figure 12: shows nucleotide sequence (SEQ ID NO 19) and corresponding amino acid sequence (SEQ ID NO 20) of the open reading frame of the region comprised between the NcoI and SacI sites of plasmid pFAJ3109. The amino acids corresponding to mature DmAMP1 are underlined.

Figure 13: shows the Dm-AMP1 expression levels (as % of total soluble protein) of a series of transgenic individual plants transformed with construct pFAJ3105 and a series of transgenic individuals transformed with construct pFAJ3109.

Figure 14: shows RP-HPLC analysis on a C8-silica column of crude extracts from leaves transformed with construct pFAJ3105 (A) or pFAJ3106 (B). Extracts were prepared as described in Materials and Methods. The column was eluted with a gradient of acetonitrile in 0.1 % TFA (0-35 min. 15 % - 50 % acetonitrile in 0.1 % TFA). The eluate was monitored on-line for measurement of the absorbance at 214 nm (top trace), fractionated, and subjected to Elisa assays for DmAMP1 (lower bar graph, black bars) and RsAFP2 (lower bar graph, white bars). The elution position of authentic DmAMP1 and RsAFP2 are indicated with arrows on the A_{214} chromatograms.

Figure 15: shows the results of reverse phase chromatography (RPC) of the extracellular fluid fraction of Arabidopsis plants transformed with construct 3105 (line 14). RPC was performed on a C8-silica column (Microsorb-MV, 4.6 x 250 mm, Rainin) equilibrated with 0.1 % trifluoroacetic acid (TFA). After loading the column was eluted at a flow rate of 1 ml/min for 20 min with 0.1 % TFA, whereafter a 35 min linear gradient was applied from 15 to 50 % acetonitrile in 0.1 % TFA. Absorbance (full line) was measured on-line at 280 nm and acetonitrile concentration (dashed line) was measured on-line with a conductivity monitor. Fractions were collected and assessed for DmAMP1-CRP and RsAFP2-CRP using ELISA assays. Peak numbers in bold indicate presence of DmAMP1-CRP, peak numbers in italic indicate presence of RsAFP2-CRP.

Figure 16: shows the results of RPC of an extract of Arabidopsis plants transformed with construct 3105 (line 14). Samples were two different fractions from IEC showing presence of either DmAMP1-CRPs or RsAFP2-CRPs, namely those fractions eluting between 0.17 – 0.33 M NaCl (A), and 0.33 – 0.49 M NaCl (B). RPC was performed as in the legend to Figure 14. Absorbance (full line) was measured on-line at 280 nm and acetonitrile concentration (dashed line) was measured on-line with a conductivity monitor. Fractions were collected and assessed for DmAMP1-CRP or RsAFP2-CRP using ELISA assays. Peak numbers in bold indicate presence of DmAMP1-CRP, peak numbers in italic indicate presence of RsAFP2-CRP.

Figure 17: shows the amino acid sequence of the polyprotein precursors encoded by constructs pFAJ3105, pFAJ3106 and pFAJ3108. Dashes indicate omission from the full sequence for sake of brevity. The sequence in italic is the DmAMP1 leader peptide, the underlined sequence is mature DmAMP1, the bold sequence is the linker peptide, the double underlined

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sequence is mature RsAFP2. Arrows indicate processing sites according to the N-terminal sequence and mass spectrometry analyses of purified DmAMP-CRPs and RsAFP2-CRPs.

Figure 18: shows the RPC of the extracellular fluid fraction of Arabidopsis plants transformed with construct pFAJ3106 (line 9). RPC was performed and fractions analysed
5   as described in the legend to figure 15. Peak numbers in bold indicate presence of DmAMP1-CRP, peak numbers in italic indicate presence of RsAFP2-CRP.

Figure 19: shows the RPC results of an extract of Arabidopsis plants transformed with construct 3108 (line 9). The sample was a fraction from IEC showing presence of either DmAMP1-CRPs or RsAFP2-CRPs, namely those fractions eluting between 0.17 – 0.33 M
10 NaCl and showing the presence of DmAMP1-CRPs. RPC was performed and fractions analysed as in the legend to Figure 15. Peak numbers in bold indicate presence of DmAMP1-CRP.

Figure 20: is a schematic representation of the coding region of constructs pFAJ3105, pFAJ3343, pFAJ3344, pFAJ3345, pFAJ3346, and pFAJ3369. Full arrowheads indicate
15 experimentally determined cleavage sites. Open arrowheads indicate presumed cleavage sites. Abbreviations: SP DmAMP1: signal peptide region of DmAMP1 (see figure 1); DmAMP1: mature protein region of DmAMP1 (see figure 1); RsAFP2: mature protein region of RsAFP2 (Terras et al. 1995, Plant Cell, 7, 573-588). Linker peptide sequences are shown in full (SEQ ID NOS 3, 29, 21-24 respectively).

20 Figure 21: is a schematic representation of the coding region of constructs pFAJ3367 with linker peptide of SEQ ID NO 24. Abbreviations: SP DmAMP1: signal peptide region of DmAMP1 (see figure 1); DmAMP1: mature protein region of DmAMP1 (see figure 1); RsAFP2: mature protein region of RsAFP2 (Terras et al. 1995, Plant Cell, 7, 573-588); HsAFP1: mature protein region of HsAFP1 (Osborn et al. 1995, FEBS Lett. 368, 257-262);
25 AceAMP1; mature protein region of AceAMP1 (Cammue et al. 1995, Plant Physiol. 109, 445-455).

Figure 22: is a schematic representation of the coding region of constructs pFAJ3106-2, pFAJ3107-2, and pFAJ3108-2. Abbreviations: SP DmAMP1: signal peptide region of DmAMP1 (see figure 1); DmAMP1: mature protein region of DmAMP1 (see figure 1);

RsAFP2: mature protein region of RsAFP2 (Terras et al. 1995, Plant Cell, 7, 573-588); RS Kex2p: recognition sequence (IGKR) of the Kex2 protease (Jiang and Rogers, 1999, Plant J., 18, 23-32); AcAMP1: mature protein region of AcAMP1 (De Bolle et al. Plant Mol Biol, 31, 997-1008). The linker propeptide sequences are shown in full as SEQ ID NOS 25, 26 and 27
5   respectively.

Figure 23: is a schematic representation of the coding region of constructs pFAJ3368 and pFAJ3370. Open arrowheads indicate presumed cleavage sites. Abbreviations: SP DmAMP1: signal peptide region of DmAMP1 (see figure 1); DmAMP1: mature protein region of DmAMP1 (see figure 1); RsAFP2: mature protein region of RsAFP2 (Terras et al.
10   1995, Plant Cell, 7, 573-588); 2A sequence: cleavage recognition site of the Foot and Mouth Disease Virus polyprotein. The linker propeptide sequence is shown in full as SEQ ID NO 28.

Figure 24: shows nucleotide sequence (SEQ ID NO 30) and corresponding amino acid sequence (SEQ ID NO 31) of the open reading frame of the region comprised between the
15   NcoI and SacI sites of plasmid pFAJ3343. The amino acids corresponding to mature DmAMP1 and mature RsAFP2 are underlined and double-underlined, respectively. The amino acids corresponding to the internal linker peptide are in bold (SEQ ID NO 29).

Figure 25: shows the nucleotide sequence (SEQ ID NO 32) and corresponding amino acid sequence (SEQ ID NO 33) of the open reading frame of the region comprised between the
20   NcoI and SacI sites of plasmid pFAJ3344. The amino acids corresponding to mature DmAMP1 and mature RsAFP2 are underlined and double-underlined, respectively. The amino acids corresponding to the internal linker peptide are in bold (SEQ ID NO 21).

Figure 26: shows the nucleotide sequence (SEQ ID NO 34) and corresponding amino acid sequence (SEQ ID NO 35) of the open reading frame of the region comprised between the
25   NcoI and SacI sites of plasmid pFAJ3345. The amino acids corresponding to mature DmAMP1 and mature RsAFP2 are underlined and double-underlined, respectively. The amino acids corresponding to the internal linker peptide are in bold (SEQ ID NO 22).

Figure 27: shows the nucleotide sequence (SEQ ID NO 36) and corresponding amino acid sequence (SEQ ID NO 38) of the open reading frame of the region comprised between the

NcoI and SacI sites of plasmid pFAJ3346. The amino acids corresponding to mature DmAMP1 and mature RsAFP2 are underlined and double-underlined, respectively. The amino acids corresponding to the internal linker peptide are in bold (SEQ ID NO 23).

Figure 28: shows the nucleotide sequence (SEQ ID NO 38) and corresponding amino acid sequence (SEQ ID NO 39) of the open reading frame of the region comprised between the NcoI and SacI sites of plasmid pFAJ3369. The amino acids corresponding to mature DmAMP1 and mature RsAFP2 are underlined and double-underlined, respectively. The amino acids corresponding to the internal linker peptide are in bold (SEQ ID NO 24) .

Figure 29: shows the nucleotide sequence and corresponding amino acid sequence of the open reading frame of the region comprised between the NcoI and SacI sites of plasmid pFAJ3367. The amino acids corresponding to mature DmAMP, mature RsAFP2, mature HsAFP1 and mature AceAMP1 are underlined, double-underlined, dashed-underlined and dotted-underlined, respectively. The amino acids corresponding to the internal linker peptides are in bold (SEQ ID NO 24).

Figure 30: shows the nucleotide sequence (SEQ ID NO 42) and corresponding amino acid sequence (SEQ ID NO 43) of the open reading frame of the region comprised between the NcoI and SacI sites of plasmid pFAJ3106-2. The amino acids corresponding to mature DmAMP1 and mature RsAFP2 are underlined and double-underlined, respectively. The amino acids corresponding to the internal linker peptide are in bold (SEQ ID NO 4).

Figure 31: shows the nucleotide sequence (SEQ ID NO 44) and corresponding amino acid sequence (SEQ ID NO 45) of the open reading frame of the region comprised between the NcoI and SacI sites of plasmid pFAJ3107-2. The amino acids corresponding to mature DmAMP1 and mature RsAFP2 are underlined and double-underlined, respectively. The amino acids corresponding to the internal linker peptide are in bold (SEQ ID NO 6).

Figure 32: shows the nucleotide sequence (SEQ ID NO 46) and corresponding amino acid sequence (SEQ ID NO 47) of the open reading frame of the region comprised between the NcoI and SacI sites of plasmid pFAJ3108-2. The amino acids corresponding to mature DmAMP1 and mature RsAFP2 are underlined and double-underlined, respectively. The amino acids corresponding to the internal linker peptide are in bold (SEQ ID NO 7).

Figure 33: shows the nucleotide sequence (SEQ ID NO 48) and corresponding amino acid sequence (SEQ ID NO 49) of the open reading frame of the region comprised between the NcoI and SacI sites of plasmid pFAJ3370. The amino acids corresponding to mature DmAMP1 and mature RsAFP2 are underlined and double-underlined, respectively. The linker sequence is indicated in bold type (SEQ ID NO 28) with the amino acids corresponding to the 2A sequence indicated in bold italic.

Figure 34: shows the nucleotide sequence (SEQ ID 48) and corresponding amino acid sequence (SEQ ID NO 49) of the open reading frame of the region comprised between the NcoI and SacI sites of plasmid pFAJ3368. The amino acids corresponding to mature DmAMP1 and mature RsAFP2 are underlined and double-underlined, respectively. The linker sequence is indicated in bold type with amino acids corresponding to the 2A sequence are indicated in bold italic.

The following Examples illustrate the invention.

Example 1

Cloning of DmAMP1 cDNA and DmAMP1 gene

Cloning procedures and polymerase chain reaction (PCR) procedures were performed following standard protocols (Sambrook et al., 1989, Molecular Cloning: a laboratory manual, 2nd edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY). A cDNA library was constructed from near-dry seeds collected from flowers of Dahlia merckii. Total RNA was purified from the seeds using the method of Jepson I. et al. (1991, Plant Mol. Biol. Reporter 9, 131-138). 0.6 mg of total RNA was obtained from 2 g of D. merckii seed. PolyATract magnetic beads (Promega) were used to isolate approximately 2 μg poly-A+ RNA from 0.2 mg of total RNA.

The poly-A+ RNA was used to construct a cDNA library using a ZAP-cDNA synthesis kit (Stratagene). Following first and second strand synthesis, cDNAs were ligated with vector DNA. After phage assembly using Gigapack Gold (Stratagene) packaging extracts, approximately 1 x 10^5 plaque forming units (pfu) were obtained.

Using oligonucleotides AFP-5 (5'-TG(T,C)GANAANGCN(A,T)(G,C)NAA(A,G)ACNTGG)

(SEQ ID NO 13) based on the N-terminal sequence CEKASKTW (SEQ ID NO 14) of DmAMP1, Osborn R.W. *et al.*, 1995, FEBS Lett. 368, 257-262) and AFP-3EX (5'-CA(A,G)TT(A,G)AANTANCANAAA(A,G) CACAT) (SEQ ID NO 52) based on the C-terminal sequence MCFCYFNC (SEQ ID NO 53) of DmAMP1) and genomic DNA isolated

5   from *D. merckii* leaves, a 144 bp PCR product was produced and isolated from an agarose gel. The PCR product was cloned into pBluescript. The insert of 10 transformants were sequenced.   The sequences represented 3 closely homologous DmAMP1-like genes one of which, PCR clone 4, encoded the observed mature DmAMP1. The 144 bp PCR product mixture labelled with ^{32}-P CTP was used to probe Hybond N (Amersham) filter lifts made

10   from plates containing a total of 6 x 10^4 pfu of the cDNA library. Thirty potentially positive signals were observed. 22 plaques were picked and taken through two further rounds of screening. After in vivo excision 13 clones were characterised by DNA sequencing. Four classes of DmAMP related peptides were encoded by the 13 cDNA clones. Three versions of the DmAMP mature protein region were represented in the four classes. One of

15   the classes (Dm2.5 type) contained a mature protein region which may correspond to DmAMP2 (Osborn R.W. *et al.*, 1995, FEBS Lett. 368, 257-262). None of the cDNAs encoded a mature protein region equivalent to the observed mature DmAMP1 peptide sequence.

       Using the sequence of PCR clone 4 (above) and information from the N- and C-terminal ends of the peptides deduced from cDNA sequences, two pairs of oligonucleotides

20   were designed for amplification of a gene encoding DmAMP1. Genomic DNA from *D. merckii* was used in a PCR reaction with oligonucleotides MATAFP-5P (5'-ATGGC(C,G)AAN(A,C)(A,G)NTC (A,G)GTTGCNTT) (SEQ ID NO 66) and MATAFP-5 (5'- AAACACATGTGTTTCCCATT) (SEQ ID NO 54), the PCR product was cloned into pBluescript and clones were sequenced. A clone containing the 5' half of a DmAMP1 gene

25   was identified. Genomic DNA from *D. merckii* was used in a PCR reaction with MATAFP-3 (5'- AGCGTGTCATGTGCGTAAT) (SEQ ID NO 55) and DM25MAT-3 (5'- TAAAGA AACCGACCCTTTCACGG) (SEQ ID NO 56), the PCR product was cloned into pBluescript and clones were sequenced. A clone containing the 3' half of a DmAMP1 gene was identified. The 5' and 3' sections of the mature gene were combined to assemble the sequence of the

30   coding region of the DmAMP1 gene (Figure 1).

The DmAMP1 gene encodes a precursor with a 28 amino acids leader peptide, a 50 amino acids mature protein and a 40 amino acids C-terminal propeptide. The open reading frame is interrupted by a 92 bp intron located within the leader peptide region.

To eliminate the intron from the DmAMP1 gene sequence and to allow cloning of the

5   DmAMP1 encoding region, either with or without the C-terminal propeptide region, into an expression cassette vector, two PCR reactions were carried out with respectively the primer sets DMVEC-3 (5'- ATGCATCCATGGTGAATCGGTCGGTTGCGTTCTCCGCGTTCGTT CTGATCCTTTTCGTGCTCGCCATCTCAGATATCGCATCCGTTAGTGGAGAACTATG CGAGAAA) (SEQ ID NO 57) and DMVEC-2 (5'-

10   AAACCGACCGAGCTCACGGATGTTCAACGTTTGGA AC) (SEQ ID NO 58), and DMVEC-3 and DMVEC-4 (5'- AGCAAGCTTTTCGGGAGCTCAACAATTGA AGTAA)(SEQ ID NO 59). DMVEC-3 primes at the top strand of the DmAMP1 gene, corresponds to the leader peptide region without the intron and introduces an *Nco*I site at the translation start. DMVEC-2 primes at the bottom strand of the DmAMP1 gene at the 3'-end

15   of the C-terminal propeptide region and introduces a *Sac*I site behind the translation stop codon. DMVEC-4 primes at the bottom strand of the DMAMP1 gene at the 3' end of the mature protein region, fuses a stop codon behind this region and introduces a *Sac*I site behind the stop codon.

Both PCR products were cut with *Nco*I and *Sac*I which cleaved the PCR products in

20   two fragments due to an internal *Nco*I site in the mature protein region. The resulting *Nco*I-*Sac*I and *Nco*I-*Nco*I fragments were cloned sequentially in plasmid pMJB1. pMJB1 is an expression cassette vector containing in sequence a *Hin*dIII site, the enhanced cauliflower mosaic 35S RNA (CaMV35S) promoter (Kay R. *et al.*, 1987, Science 236, 1299-1302), a *Xho*I site, the 5' untranslated leader sequence of tobacco mosaic virus (TMV) (Gallie D.R. and

25   Walbot V., 1992, Nucl. Ac. Res. 20, 4631-4638) a polylinker including *Nco*I, *Sma*I, *Kpn*I and *Sac*I sites, the 3' untranslated terminator region of the *Agrobacterium tumefaciens* nopaline synthase gene (Bevan M.W. *et al.*, 1983, Nature 304, 184-187) and an *Eco*RI site. The resulting plasmids were termed pDMAMPE (leader peptide region, mature protein region and C-terminal propeptide region) and pDMAMPD (leader peptide region and mature protein

30   region), respectively. The coding regions were verified by DNA sequencing.

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Example 2

Construction of plant transformation vectors

To explore the possibility of expressing polyprotein precursor genes in plants, four different plant transformation vectors were made with the aim to co-express two different cysteine-rich plant defensins with antifungal properties, namely RsAFP2 and DmAMP1. The polyprotein precursor regions of these constructs all featured a leader peptide region derived from the DmAMP1 cDNA, the mature protein domain of DmAMP1, an internal propeptide region, and the mature protein domain of RsAFP2. The four constructs differed only in the internal propeptides (Figure 2):

- construct 3105 has one of the IbAMP internal propeptides as a propeptide separating DmAMP1 and RsAFP2.

- construct 3106 has a propeptide consisting of a part of the DmAMP1 propeptide and a putative subtilisin-like protease processing site (IGKR) (SEQ ID NO 67) at its C-terminus.

- construct 3107 is identical to construct 3106 except that the entire DmAMP1 propeptide was taken.

- construct 3108 has a propeptide consisting of the AcAMP2 propeptide and a putative subtilisin-like protease processing site (IGKR) at its C-terminus.

The rationale behind constructs 3106, 3107 and 3108 is based on our observations that the C-terminal propeptides of AcAMP2 and DmAMP1 are cleaved off at their N-terminus when expressed as AcAMP2- and DmAMP1-preproproteins in tobacco, respectively, while this processing event does not detract the mature proteins from being sorted to the apoplast (De Bolle et al., 1996, Plant Mol. Biol. 31, 993-1008; R.W. Osborn and S. Attenborough, personal communication). This infers that the processing enzymes are either in the secretory pathway or in the apoplast. On the other hand, C-terminal cleavage of the internal propeptide in these constructs should be executed by a subtilisin-like protease, a member of which in yeast (Kex2) is known to occur in the Golgi apparatus (Wilcox C.A. and Fuller R.S., 1991, J. Cell. Biol. 115, 297), while a member in tomato occurs in the apoplast (Tornero P. et al., 1997, J. Biol. Chem. 272, 14412-14419). Proteins deposited in the apoplast, the preferred deposition site for antimicrobial proteins engineered in transgenic plants (Jongedijk E. et al., 1995, Euphytica 85, 173-180; De Bolle et al., 1996, Plant Mol. Biol. 31, 993-1008) are normally synthesized via the secretory pathway, encompassing the Golgi apparatus.

A construct was also made for expression of only DmAMP1 (construct 3109, figure 7).

Schematic representations of the plant transformation vectors prepared, pFAJ3105, pFAJ3106, pFAJ3107, pFAJ3108 and pFAJ3109, are shown in Figures 3 to 7, respectively. The nucleotide sequences comprised between the *Xho*I and *Sac*I sites of these plasmids, which

5   encompass the regions encoding antimicrobial proteins, are presented in Figures 8 to 13. The regions comprised between the *Xho*I and *Sac*I sites of plasmid pFAJ3105 (shown in Figure 8) was constructed following the two-step recombinant PCR protocol of Pont-Kindom G.A.D. (1994, Biotechniques 16, 1010-1011). Primers OWB175 (5'AGGAAGTTCATTTCATTTGG) and (SEQ ID NO 68), OWB278 (5'-

10   GCCTTTGGCACAACTTCTGT CCTGGCTCCACGTCCTCTGGGGTAGCCACCTCGTCAGCAGCGTTGGAACAATTGA AGTAACAGAAACAC) (SEQ ID NO 60) were used in a first PCR reaction with plasmid pDMAMPE (see above) as a template. The second PCR reaction was done using as a template plasmid pFRG4 (Terras F.R.G. *et al.*, 1995, Plant Cell 7, 573-588) and as primers a mixture of

15   the PCR product of the first PCR reaction, primer OWB175 and primer OWB172 (5'TTA<u>GAGCTC</u>CTATTAACAAGGAAAGTAGC (SEQ ID NO 61), *Sac*I site underlined). The resulting PCR product was digested with *Xho*I and *Sac*I and cloned into the expression cassette vector pMJB1 (see above). The expression cassette in the resulting plasmid, called pFAJ3099, was digested with *Hind*III (flanking the 5' end of the CaMV35S promoter) and

20   *Eco*RI (flanking the 3' end of the nopaline synthase terminator) and cloned in the corresponding sites of the plant transformation vector pGPTVbar (Becker D. *et al.*, 1992, Plant Mol. Biol. 20, 1195-1197) to yield plasmid pFAJ3105.

Plasmids pFAJ3106, pFAJ3107 and pFAJ3108 were constructed analogously except that primer OWB278 in the first PCR reaction was replaced by the following primers,

25   respectively: OWB279 (5'- GCCTTTGGCACAACTTCTGCCTCTTTCCGATGAGTTGTTCGGCTTT AAGTTTGTC); (SEQ ID NO 62), OWB303 (5'-GCCTTTGGCACAACTTCTGCCTCTTTCCG ATCGGATGTTCAACGTTTGGAACC) (SEQ ID NO 63) ; OWB304 (5'- GCCTTTGGCACAACTTCTGCCT

30   CTTTCCGATAGTTTTGGTGGCAGCAACATCAGCTTGGTGATCCACAGTAGTACTGG CACAATTGAAGTAACAGAAACAC) (SEQ ID NO 64).

Plasmid pFAJ3109 was constructed by cloning the *Hind*III-*Eco*RI fragment of plasmid pDMAMPD (see above) into the corresponding sites of plant transformation vector pGPTVbar (see above).

5 **Example 3**

Plant transformation

Arabidopsis thaliana ecotype Columbia-O was transformed using recombinant *Agrobacterium tumefaciens* by the inflorescence infiltration method of Bechtold N. *et al.* (1993, C.R. Acad. Sci. 316, 1194-1199). Transformants were selected on a sand/perlite
10 mixture subirrigated with water containing the herbicide Basta (Agrevo) at a final concentration of 5 mg/l for the active ingredient phosphinothricin.

Example 4

Assays for target proteins including Elisa assays and protein assays
15 Antisera were raised in rabbits injected with either RsAFP2 (purified as described in Terras F.R.G. *et al.*, 1992, J. Biol. Chem. 267, 15301-15309) or DmAMP1 (purified as in Osborn R.W. *et al.*, 1995, FEBS Lett. 368, 257-262). ELISA assays were set up as competitive type assays essentially as described by Penninckx I.A.M.A. *et al.* (1996, Plant Cell 8, 2309-2323). Coating of the ELISA microtiter plates was done with 50 ng/ml RsAFP2
20 or DmAMP1 in coating buffer. Primary antisera were used as 1000- and 2000-fold diluted solutions (DmAMP1 and RsAFP2, respectively) in 3 % (w/v) gelatin in PBS containing 0.05 % (v/v) Tween 20.

Total protein content was determined according to Bradford (1976, Anal. Biochem. 72, 248-254) using bovine serum albumin as a standard.
25 Arabidopsis leaves were homogenized under liquid nitrogen and extracted with a buffer consisting of 10 mM NaH_2PO_4, 15 mM Na_2HPO_4, 100 mM KCl, 1.5 M NaCl. The homogenate was heated for 10 min at 85°C and cooled down on ice. The heat-treated extract was centrifuged for 15 min at 15 000 x g and was injected on a reserved phase high pressure liquid chromatography column (RP-HPLC) consisting of C8 silica (0,46 cm x 25 cm; Rainin)
30 equilibrated with 0.1 % (v/v) trifluoroacetic acid (TFA). The column was eluted at 1 ml/min in a linear gradient in 35 min from 15 % to 50 % (v/v) acetonitrile in 0.1 % (v/v) TFA. The

eluate was monitored for absorbance at 214 nm, collected as 1 ml fractions, evaporated and finally redissolved in water. The fractions were tested by ELISA assays.

Example 5

5   ## Preparation of intracellular extract

Intercellular fluid was collected from Arabidopsis leaves by immersing the leaves in a beaker containing extraction buffer (10 mM NaH₂PO₄, 15 mM Na₂HPO₄, 100 mM KCl, 1.5 M NaCl). The beaker with the leaves was placed in a vacuum chamber and subjected to six consecutive rounds of vacuum for 2 min followed by abrupt release of vacuum. The infiltrated
10   leaves were gently placed in a centrifuge tube on a grid separated from the tube bottom. The intercellular fluid was collected from the bottom after centrifugation of the tubes for 15 min at 1800 x g. The leaves were resubjected to a second round of vacuum infiltration and centrifugation and the resulting (extracellular) fluid was combined with that obtained after the first vacuum infiltration. After this step the leaves were extracted in a Phastprep
15   (BIO101/Savant) reciprocal shaker and the extract clarified by centrifugation (10 min at 10,000 x g) and the resulting supernatant considered as the intracellular extract.

Expression levels of DmAMP1 and RsAFP2 were analysed in leaves taken from a series of T1 transgenic Arabidopsis plants resulting from transformation with the constructs described above. The results of the expression analyses based on Elisa assays as described
20   above are presented in Table 1.

Table 1: Expression levels of Dm-AMP1 and Rs-AFP2 in transgenic Arabidopsis lines

construct	line	Expression level of Dm-AMP1 (%)	expression level of Rs-AFP2 (%)	construct	line	Expression level of Dm-AMP1 (%)	expression level of Rs-AFP2 (%)
3105	1	0,77	0,29	3107	1	0,04	0,04
	2	1,13	0,22		2	0,75	0,42
	3	0,48	0,20		3	0,14	0,13
	4	0,005	<0,001		4	0,01	0,01
	5	0,36	0,05		5	0,27	0,29
	6	0,99	0,25	3108	1	0,47	0,10
	7	0,60	0,09		2	3,00	0,53
	8	0,13	<0,001		3	0,91	0,24
	9	0,25	0,08		4	2,04	0,22
	10	4,15	0,85		5	0,17	0,04
	11	1,35	0,35		6	0,55	0,05
	12	0,24	0,07		7	0,16	0,11
	13	4,43	0,91		8	0,05	0,02
	14	1,18	0,24		9	0,45	0,02
	15	0,68	0,17	3109	1	0,19	nd
	16	0,49	0,07		2	0,05	nd
3106	1	0,10	0,001		3	0,02	nd
	2	1,82	0,008		4	0,20	nd
	3	0,68	0,20		5	0,10	nd
	4	1,15	0,38		6	0,06	nd
	5	0,20	0,10		7	0,07	nd
	6	0,10	0,05		8	0,003	nd
	7	0,40	0,17		9	0,18	nd
	8	2,64	0,50				
	9	0,40	0,15				
	10	0,21	0,07				
	11	0,06	0,03				
	12	0,24	0,09				

5   In the above Table "nd" indicates not done.

Most of the tested lines transformed with the polyprotein constructs 3105, 3106, 3107 and 3108 clearly expressed both DmAMP1-CRPs (DmAMP1-crossreactive proteins) and RsAFP2-CRPs (Rs-AFP2-crossreactive proteins). There was generally a good correlation between DmAMP1-CRP and RsAFP2-CRP levels. However, the RsAFP2-CRP levels were generally

5   2 to 5-fold lower than the DmAMP1-CRP levels. The Elisa assays for measuring the RsAFP2-CRPs in the extracts are, however, less reliable than those for the Dm-AMP1-CRPs. In Rs-AFP2 Elisa assays, dilutions of extracts of transgenic plants yielded dose-response curves that deviated from those obtained for dilutions of standard solutions containing authentic Rs-AFP2, indicating that the majority of the Rs-AFP2 -CRPs in the extracts were

10   imunologically not identical to RsAFP2 itself. Deviations from RsAFP2 standard dose-response curves were much more pronounced for extracts from plants transformed with constructs 3106, 3107, and 3108 than for those of plants transformed with 3105.

None of the extracts showed deviations from Dm-AMP1 standards in dose response curves in Dm-AMP1 Elisa assays. The DmAMP-CRP levels in the lines transformed with

15   the polyprotein constructs 3105, 3106, 3107 or 3108 were generally much higher compared to those in the line transformed with the single protein construct 3109. This is also illustrated in Figure 13 where DmAMP1-CRP expression levels are compared for plants transformed with the polyprotein construct 3105 and plants transformed with the single protein construct 3109. Expression levels as high as 4% of total protein (e.g. DmAMP1-CRP level in lines 3105-15

20   and 3105 -18, see table 1) have so far never been reported in the literature for a peptide expressed in transgenic plants. Hence, the use of polyprotein constructs appears to result in markedly enhanced expression, which is an unexpected finding.

Example 6

25   **Separation of proteins processed from polyprotein precursors**
A transgenic line was selected among each of the populations transformed with either construct 3105 (line 1) or 3106 (line 2) and the selected lines were further bred to obtain plants homozygous for the transgenes. In order to analyse whether DmAMP1 and RsAFP2 were correctly processed in these lines, extracts from the plants were prepared as described in

30   Example 1 and separated by RP-HPLC on a C8-silica column. Fractions were collected and

assessed for presence of compounds cross-reacting with antibodies raised against either DmAMP1 or RsAFP2 using Elisa assays as described in Example 4.

As shown in figure 15. DmAMP1- CRPs eluted at a position identical or very close to that of authentic DmAMP1 in the line transformed with construct 3105 as well as in that transformed

5   with construct 3106. Likewise, RsAFP2-CRPs were detected in both the construct 3105 and 3106 lines at an elution position identical or very close to that of authentic RsAFP2. None of the fractions reacted with both the anti-DmAMP1 and anti-RsAFP2 antibodies, indicating that an uncleaved fusion protein was not present in the extracts. No cross-reacting compounds were observed in a non-transformed line.

10   Thus it appears that the primary translation products of the transcription units of construct 3105 (IbAMP internal propeptide as linker peptide) and construct 3106 (partial DmAMP1 C-terminal propeptide with subtilisin-like protease site as a linker peptide) are somehow processed to yield separate DmAMP1-CRPs and RsAFP2-CRPs that appear to be identical or very closely related to DmAMP1 and RsAFP2, respectively, based on their

15   chromatographic behavior.

Example 7

Analysis of the subcellular location of coexpressed plant defensins

In order to determine whether the coexpressed plant defensins are either secreted

20   extracellularly or deposited intracellularly, extracellular fluid and intracellular extract fractions were obtained from leaves of homozygous transgenic Arabidopsis lines transformed with either constructs 3105 (line 2), 3106 (line 2) or 3108 (line 12). The cytosolic enzyme glucose-6-phosphate dehydrogenase was used as a marker to detect contamination of the extracellular fluid fraction with intracellular components. As shown in Table 2, glucose-6-phosphate

25   dehydrogenase was partitioned in a ratio of about 80/20 between intracellular extract fractions and extracellular fluid fractions. In contrast, the majority of DmAMP1-CRP and RsAFP2-CRP content in all transgenic plants tested was found in the extracellular fluid fractions. These results indicate that both plant defensins released from the polyprotein precursors are deposited primarily in the apoplast. Hence, all processing steps that result in cleavage of the

30   polyprotein structure must occur either in the apoplast or along the secretory pathway i.e. in

the endoplasmic reticulum, the Golgi apparatus or in vesicles trafficking between Golgi and apoplast.

5   <u>Table 2</u>:   Relative abundance of glucose-6-phosphate dehydrogenase activity (GPD), DmAMP1 and RsAFP2 in the extracellular fluid (EF) and intracellular extract (IE) fractions obtained from transgenic Arabidopsis plants.

Construct	Relative abundance[1] (%) of					
	GPD		DmAMP1		RsAFP2	
	EF	IE	EF	IE	EF	IE
pFAJ3105	17	83	93	7	92	8
pFAJ3106	17	83	94	6	60	40
pFAJ3108	20	80	98	2	75	25

[1]Relative abundance is expressed as % of the sum of the contents in the EF and IE fractions.

10   **Example 8**

Purification of proteins processed from polyprotein precursor construct 3105

Transgenic line 14 from the population transformed with construct 3105 was further bred to obtain plants homozygous for the transgene. The DmAMP1-CRPs and RsAFP2-CRPs were purified by reversed phase chromatography from extracellular fluid prepared from leaves of

15   this line. To this end, leaves were vacuum infiltrated with a buffer containing 50 mM MES (pH6) and a mixture of protease inhibitors (1 mM phenylmethylsulfonylfluoride, 1mM N-ethylmaleimide, 5mM EDTA and 0.02 mM pepstatin A), and the extracellular fluid collected by centrifugation. Using this procedure homogenization and hence exposing DmAMP1-CRPs and RsAFP2-CRPs to compartimentalized proteases was avoided. The collected

20   extracellular fluid was analyzed by RP-HPLC on a C8-silica column (Microsorb-MV, 4.6 x 250 mm, Rainin) and the fractions tested for presence of DmAMP1-CRPs and RsAFP2-CRPs by Elisa using antibodies raised against DmAMP1 and RsAFP2, respectively. The result of this analysis for the Arabidopsis transgenic line 14 transformed with construct 3105 is shown in figure 15. DmAMP1-CRPs eluted in two peaks, the latter of which eluted at a

position very close to that of authentic DmAMP1. RsAFP2-CRPs were found in a single peak that was well separated from the DmAMP1-CRP peaks and eluted at a position very close to that of authentic RsAFP2. None of the fractions reacted with both the anti-DmAMP1 and anti-RsAFP2 antibodies, indicating that an uncleaved fusion protein was absent from the extracellular fluid. Based on comparison of the peak areas of the DmAMP1-CRPs and RsAFP2-CRPs with those of a series of standards consisting of authentic Dm-AMP1 and RsAFP2, respectively, it was judged that the extract for the line transformed with construct 3105 contained about equal amounts of DmAMP1-CRPs and RsAFP2-CRPs. This indicates that cleavage of the polyprotein precursor in this line results in about equimolar amounts of DmAMP1-CRPs and RsAFP2-CRPs. Very similar chromatograms were obtained upon analysis of extracellular fluid prepared from transgenic line 2 (results not shown), indicating that the chromatographic pattern of DmAMP1-CRPs and RsAFP2-CRPs is independent from the transgenic line tested.

To test whether the purification procedure based on extracellular fluid preparation reflects the true composition in DmAMP-CRPs and RsAFP2-CRPs of the transgenic Arabidopsis leaves, an alternative purification procedure was developed starting from a crude leaf extract. To this end, leaves were homogenized under liquid nitrogen and extracted with 50 mM MES (pH6) containing a mixture of protease inhibitors (1 mM phenylmethylsulfonylfluoride, 1mM N-ethylmaleimide, 5mM EDTA and 0.02 mM pepstatin A). The homogenate was cleared by centrifugation (10 min at 10000 x g). The supernatant was then fractionated by ion exchange chromatography (IEC) and subsequently by reversed phase chromatography (RPC). After each separation, fractions were collected and assessed for DmAMP-CRPs and RsAFP2-CRPs using two different Elisa assays with antibodies raised against DmAMP1 and RsAFP2, respectively. IEC was performed by passing the extract over a cation exchange column (Mono S, 5 x 50 mm, Pharmacia) at pH 6. When the column was eluted with a linear gradient of 0 to 0.5 M NaCl in 50 mM N-morpholino ethane sulfonic acid (MES) at pH 6, DmAMP1-CRPs were detected in fractions eluting between 0.17 and 0.33 M NaCl, while RsAFP2-CRPs eluted between 0.24 and 0.49 M NaCl. Fractions containing either DmAMP1-CRPs or RsAFP2-CRPs were pooled into two fractions (0.17 to 0.33 M NaCl; and 0.33 to 0.49 M NaCl) which were each subjected to RPC on a C8-silica column (Microsorb-MV, 4.6 x 250 mm, Rainin) eluted with a linear gradient

of acetonitrile (Figure 16). DmAMP1-CRPs eluted in two peaks. the latter of which eluted at a position very close to that of authentic DmAMP1. RsAFP2-CRPs were found in a single peak that was well separated from the DmAMP-CRP peaks and eluted at a position very close to that of authentic RsAFP2. Again, none of the fractions reacted with both the anti-DmAMP1 and anti-RsAFP2 antibodies, indicating that an uncleaved fusion protein was not present in the extracts.

The different DmAMP1-CRPs and RsAFP2-CRPs purified from extracellular fluid were subjected to N-terminal amino acid sequence analysis (procedures as described in Cammue et al., 1992, J. Biol. Chem., 2228-2233) as well as to MALDI-TOF (matrix-assisted laser desorption ionization-time of flight) mass spectrometry (Mann and Talbo, 1996, Curr. Opinion Biotechnol. 7, 11-19). The C-terminal amino acid was determined based on the best approximation of the predicted theoretical mass by the experimentally determined mass (Table 3). Both the minor DmAMP1-CRPs, p3105EF1, and the major DmAMP1-CRP, p3105EF2 (protein codes as in figure 15 and Table 3), had exactly the same N-terminal sequence as mature DmAMP1. p3105EF1 and p3105EF2 had masses that were consistent with the presence of a single additional serine residue at their C-terminal end compared to authentic DmAMP1. However, while the mass of p3105EF2 corresponded exactly (within experimental error) to that calculated for a DmAMP1 derivative with a C-terminal serine (hereafter called DmAMP1+S), that of p3105EF1 was in excess by about 8 dalton relative to the calculated mass for DmAMP1+S. Hence, this protein might be a DmAMP1+S derivative with reduced disulfide bridges. The RsAFP2-CRP fraction p3105EF3 represents, based on N-terminal sequence and mass data, an RsAFP2 derivative with the additional pentapeptide sequence DVEPG at its N-terminus. This protein is further referred to as DVEPG+RsAFP2. The different DmAMP1-CRPs and RsAFP2-CRPs purified from total leaf extract were analyzed in the same way. The analyses indicated that the same molecular species were present in the total leaf extract, i.e. DmAMP1+S, a putatively reduced form of DmAMP1+S, and DVEPG+RsAFP2 (Table 3 see Example 10 below).

The purified fractions containing the major processing products, DmAMP1+S and DVEPG+RsAFP2 respectively, were subjected to an antimicrobial activity test using the fungus *Fusarium culmorum* according to the procedure outlined by Cammue et al. (1992, J.

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Biol. Chem. 267, 2228-2233). The specific antimicrobial activity, expressed as protein concentration required for 50 % growth inhibition of the test organism, of purified DmAMP1+S was identical to that of authentic DmAMP1. The specific antimicrobial activity of purified DVPEG÷RsAFP2 was about 2-fold lower relative to that of authentic RsAFP2. The slight drop in specific antimicrobial activity of DVPEG+RsAFP2 is most likely due to the presence of 5 additional N-terminal amino acids. Nevertheless, our data prove that processing of the polyprotein precursors in transgenic plants can result in the release of bioactive proteins.

Analysis of the AFPs produced in transgenic plants transformed with construct 3105 reveals that the precursor is apparently processed by three cleavage steps (Figure 17):

(i) the precursor is cleaved at the C-terminal end of the leader peptide in the same way as for the authentic DmAMP1 precursor; (ii) the precursor is cleaved at the C-terminal end of the first amino acid of the linker peptide, thus releasing DmAMP1+S; (iii) the precursor is further processed at the N-terminal end of the fifth last residue of the linker peptide, thus releasing DVEPG+RsAFP2. It is not known which proteases effect the observed cleavages, nor how many different proteases are involved. Cleavages in the linker peptides might involve only endoproteinases or result from the coordinated action of endoproteinases and exopeptidases that further trim the cleavage products at their ends. Processing at the C-terminal side of the linker peptide occurs between the two acidic residues E and D. The acidic doublet might be a target sequence for a specific endoproteinase. An aspartic endoproteinase that is able to cleave between two consecutive acidic residues has previously been purified from Arabidopsis seeds (D'Hondt et al. 1993, J. Biol. Chem. 268, 20884-20891). It is worthwhile to mention that the sequence ED occurs at the very C-terminal end in five out of six internal propeptides of the IbAMP1 polyprotein precursor (Tailor et al. 1997, J. Biol. Chem. 272, 24480-24487). In one of the six internal IbAMP propeptides, more precisely the one that was used in construct 3105, the ED sequence does not occur at the C-terminal end of the propeptides but is separated by 4 amino acids from this end. Processing of this propeptide in *Impatiens balsamina* might involve cleavage of the ED sequence followed by partial N-terminal trimming of the resulting protein by an aminopeptidease.

It would be expected that an internal propeptide resembling the IbAMP1 propeptide used in construct 3105 but in which the ED dipeptidic sequence is moved to the C-terminal end of the propeptide, would result in a cleavage product with only one or no extra N-terminal amino acids in the protein located C-terminally from the internal propeptide.

5   Alternatively, another IbAMP1 propeptide which already has an ED sequence at its C-terminal end (Tailor *et al.*, 1997, J. Biol. Chem. 272, 24480-24487) or a related sequence might give a similar improvement of processing accuracy.

Example 9

10   **Purification of proteins processed from polyprotein precursor construct pFAJ3106**

Transgenic line 9 from the population of *Arabidopsis* plants transformed with construct pFAJ3106 was further bred to obtain plants homozygous for the transgene. The DmAMP1-CRPs and RsAFP2-CRPs were purified by reversed phase chromatography from leaf extracellular fluid prepared in the same way as described above in Example 8 for the line

15   transformed with construct pFAJ3105. The chromatogram of this separation is shown in Figure 18. DmAMP1-CRPs eluted in two peaks, called p3106EF1 and p3106EF2. Both fractions had the same N-terminal sequence as DmAMP1 (Table 3 see Example 10 below). The mass of p3106EF2 corresponded to that predicted for a DmAMP1 derivative with an additional lysine. We therefore conclude that it represents the cleavage product of the

20   precursor cleaved at the signal peptide cleavage site and C-terminally behind the first residue (lysine) of the linker peptide; This protein is further referred to as DmAMP1+K.

The RsAFP2-CRP fraction was found by N-terminal amino acid sequencing to start by the sequence LIGKRQK. Hence, this protein, called QLIGKR+ RsAFP2, is derived from cleavage of the precursor N-terminally from the sixth last residue (glutamine) of the linker

25   peptide. The proposed cleavage steps involved in processing of the precursor of construct pFAJ3106 are shown in Figure 17.

Example 10

Purification of proteins processed from polyprotein precursor construct pFAJ3108

Transgenic line 9 from the population of Arabidopsis plants transformed with construct

30   pFAJ3108 was further bred to obtain plants homozygous for the transgene. The DmAMP1-

- 39 -

CRPs and RsAFP2-CRPs were purified from a total crude leaf extract of this line, following a procedure based on IEC and RPC as described above in Example 8 for the line transformed with construct 3105. The chromatograms of the IEC and RPC separations are shown in

5   Figure 19. The IEC separation yielded two peaks containing DmAMP1-CRPs. However, no RsAFP2-CRPs could be detected in any of the eluate fractions. As RsAFP2-CRPs were clearly present in crude extracts and EF fractions of plants transformed with construct pFAJ3108 (see tables 1 and 2) the RsAFP2-CRPs must have been lost during the separation. The most likely explanation is that the RsAFP2-CRPs were not eluted from the IEC column

10   with 0.5 M NaCl, the highest concentration used in the elution gradient. Fractions containing DmAMP1-CRPs were separated by RPC, yielding two DmAMP1-CRP peak. Analysis of this fraction by N-terminal sequencing and MALDI-TOF mass determination (Table 3) revealed that it represents a DmAMP1 derivative with an additional alanine at its C-terminus (DmAMP1+A). This protein results from cleavage of the precursor at the signal

15   peptide cleavage site and C-terminally from the first residue (alanine) of the linker peptide (Figure 17).

Table 3:   Mass determined by MALDI-TOF-MS or EI-MS and N-terminal sequence
            determined by automated Edman degradation of DmAMP1-CRP and RsAFP2-
            CRP fractions purified as described in Figures 15, 16, 18 and 19. Also shown are

20   the predicted C-terminal sequence that gives best correspondence between
            experimental mass and theoretical mass.

Construct	Protein fraction (see Figures 15, 16, 18 and 19)	Mass determined by MALDI-TOF-MS	Mass determined by EI-MS	Determined N-terminal sequence (SEQ ID NOS 69-71)	Predicted C-terminal sequence (SEQ ID NOS 72-76)	Theoretical mass for predicted sequence
pFAJ3105	p3105EF1	5614 ±5	5608.3 ±1	ELCEKAS	CYFNCS	5604.25
	p3105EF2	5602 ±5	5604.9 ±1	ELCEKAS	CYFNCS	5604.25
	p3105EF3	6223 ±6	N.D.[1]	DVEPGQK	ICYFPC	6225.15
	p3105TE1	5610 ±5	N.D.	ELCEKAS	CYFNPS	5604.25
	p3105TE2	5604 ±5	N.D.	ELCEKAS	CYFNCS	5604.25
	p3105TE3	6224 ±6	N.D.	DVEPGQK	ICYFPC	6225.15
pFAJ3106	p3106EF1	N.D.	N.D.	ELCEKAS	CYFNCK	5645.34
	p3106EF2	5640 ±5	N.D.	ELCEKAS	CYFNCK	5645.34
	p3106EF3	N.D.	N.D.	LIGKRQK	ICYFPC	6295.38
pFAJ3108	p3108TE1	5583 ±5	N.D.	ELCEKAS	CYFNCA	5588.25

[1]Not determined

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Example 11

Modifications to construct pFAJ3105

From the analysis of Arabidopsis plants transformed with construct pFAJ3105 it is clear that the polyprotein precursor is indeed cleaved (see Table 3, Figure 17). However, cleavage occurs such that one amino acid from the linker peptide remains attached to the mature protein located N-terminally from the linker peptide, and that five amino acids remain attached to the mature protein located C-terminally from the linker peptide (see Figure 17). In order to reduce the number of linker peptide-derived amino acids attached to the mature proteins, which could possibly interfere with the functional properties of these mature proteins, a number of constructs have been designed in order to obtain cleavage occurring closer to (or even preferentially at) the borders of the mature proteins.

In construct pFAJ3343, the codon for the N-terminal residue of the linker peptide occurring in pFAJ3105 has been deleted. It is expected that cleavage of mature DmAMP1 will occur without addition of any amino acid from the linker peptide (Figure 20). In constructs pFAJ3344, pFAJ3345 and pFAJ3346, the codons at the carboxyl-terminal end of the linker peptide in pFAJ3105 have been modified such that the last two, four and five residues have been deleted, respectively. It is expected that the number of residues remaining attached to the N-terminal end of RsAFP2 after cleavage will be respectively three, one and zero in constructs pFAJ3344, pFAJ3345 and pFAJ3346 (Figure 20). Other constructs can be made in which the number of residues at either the N- or C-terminal end of the linker peptide region in construct pFAJ3105 is reduced.

In construct pFAJ3105 the linker peptide is derived from the fourth internal propeptide of the IbAMP precursor (Tailor R.H. *et al.*, 1997, J. Biol. Chem. 272, 24480-24487). In construct pFAJ3369, this linker peptide has been replaced by the first internal propeptide of the IbAMP precursor (Tailor R.H. *et al.*, 1997, ibid.). In the latter linker peptide the doublet of acidic residues occurs at the C-terminus. It is expected that the cleavage will occur such that only one residue will remain attached to the N-terminus of RsAFP2 (Figure 20).

Example 12

Construction of a construct for expression of a polyprotein with four mature protein domains

The polyprotein region in construct pFAJ3367 consists of the signal peptide region of DmAMP1 cDNA followed by the coding regions of four different antimicrobial peptides, each separated by the first internal propeptide region of the IbAMP precursor. The coding region for the four different antimicrobial proteins are, in order (see Figure 21):

1.   The plant defensin DmAMP1 (Osborn R.W. *et al.*, 1995, FEBS Lett. 368, 257-262)

2.   The plant defensin RsAFP2 (Terras F.R.G. *et al.*, 1995, Plant Cell 7, 573-588)

3.   The plant defensin HsAFP1 (Osborn R.W. *et al.*, 1995, FEBS Lett. 368, 257-262)

4.   The lipid transfer protein-like protein AceAMP1 (Cammue B.P.A. *et al.*, 1995, Plant Physiol. 109, 445-455)

This construct will give rise to four different mature antimicrobial proteins (DmAMP1, RsAFP2, HsAFP1 and AceAMP1), each of which secreted to the extracellular space.

Other constructs can be made other mature peptide regions and with any other linker peptide regions described above.

Example 13

Modifications to constructs pFAJ3106, pFAJ 3107 and pFAJ 3108

The polyprotein encoded by constructs pFAJ3106, pFAJ3107 and pFAJ3108 contain linker peptides with the Kex2 recognition site IGKR at their C-terminal ends. Jiang L. and Rogers J.C. (1999, Plant J. 18, 23-32) have shown that polyproteins containing a IGKR site are not or poorly cleaved in transgenic tobacco plants. Improved cleavage was observed in polyproteins in which the IGKR sequence was replaced by the IGKRIGKRIGKR (SEQ ID NO 77) sequence.

Constructs pFAJ3106-2, pFAJ3107-2 and pFAJ3108-2 are identical to constructs pFAJ3106, pFAJ3107 and pFAJ3108 except for the replacement of the IGKR coding region by a region coding for IGKRIGKRIGKR (Figure 22). Polyproteins encoded by these constructs will be efficiently cleaved both at the N-terminal end and the C-terminal end of the linker peptide.

Other constructs can be made in which the number of residues at either the N- or C-terminal end of the linker peptide region in constructs pFAJ3106, pFAJ3107 or pFAJ3108 is reduced.

Example 14

Polyprotein constructs based on hybrid linker peptides containing the 2A sequence

The foot-and-mouth disease virus (FMDV) RNA is translated as a polyprotein whose cleavage depends on a 20 amino acids sequence called the 2A sequence (Ryan and Drew

5   1994, EMBO J. 13, 928-933). Cleavage of the polyproteins joined by the 2A sequence occurs between the 19th amino acid (G) and the 20th amino acid (P) of the 2A sequence via a process which is apparently independent of processing enzymes and which might be due to improper formation of the peptide bond between G and P (Halpin et al., 1999, Plant J. 17, 453-459). Halpin C. et al. 1999 (Plant J. 17, 453-459) have shown that polyproteins

10   containing the FMDV 2A sequence as a linker peptide are efficiently cleaved when expressed in plants. One major drawback of the use of the FMDV 2A sequence as a linker peptide, however, is that cleavage does not occur at the N-terminus of the linker peptide. Hence, a relatively long stretch of 19 amino acids corresponding to the first 19 residues of the FMDV 2A sequence remains attached to the C-terminus of the mature protein. This

15   additional stretch of 19 residues may interfere with the functional properties of the protein to which it is attached.

In order to address this problem of incomplete removal of the linker peptide after cleavage, hybrid linker peptides consisting at their N-terminal part of a linker peptide described in constructs pFAJ3105, pFAJ3106, pFAJ3107 or pFAJ3108 (or a part of such

20   peptide) and at their C-terminal part of the FMDV 2A sequence (or a part of such peptide) are proposed. Examples of constructs based on this principle are constructs pFAJ3370 and pFAJ3368 (Figure 23). Construct pFAJ3370 has a polyprotein region identical to that of construct pFAJ3105 except that the linker peptide is a 29 amino acids peptide consisting of the first 9 amino acids of the fourth internal propeptide of the IbAMP precursor (Tailor R.H.

25   et al., 1997, J. Biol. Chem. 272, 24480-24487) followed by the 20 amino acids of the entire FMDV 2A sequence. Cleavage of this linker peptide should release a mature DmAMP1 with an additional serine at its C-terminus and a mature RsAFP2 with an additional proline at its N-terminus.

Construct pFAJ3368 is identical to construct pFAJ3370 except that the C-terminal

30   mature protein domain (in this case encoding RsAFP2) is replaced by a domain encoding this

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mature protein domain preceded by a signal peptide domain (in this case encoding RsAFP2
with its own signal peptide). If cleavage between G and P of the FMDV 2A sequence occurs
prior to full translocation of the polyprotein into the endoplasmic reticulum then it is
expected that construct pFAJ3368 will provide better targetting of both mature proteins to
the extracellular space in comparison to construct pFAJ3370. In this case, the secreted
mature proteins will consist of DmAMP1 with an additional serine at its C-terminus and
RsAFP2 with no added amino acids. If cleavage between G and P of the FMDV 2A
sequence occurs after translocation of the polyprotein into the endoplasmic reticulum, then it
is expected that the signal peptide attached to RsAFP2 will not be efficiently removed and in
this case construct pFAJ3370 will be preferred over pFAJ3368.